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Mate-choice copying in *Drosophila subobscura* populations

Gonçalo Faria da Silva

Dissertação
Mestrado em Biologia Evolutiva e do Desenvolvimento

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Orientadores:

Professora Susana Varela

Professora Margarida Matos

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If I was able to know every single variable that lead to this work, I would probably have a long list of thanks to make. Still, I think I can name a few of those variables and thank them for the role that they had on my thesis.

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Abstract

Mate-choice copying (MCC) by females (although the same behaviour can be found on males) occurs when they obtain information about the mating performance of a male with other females, increasing or decreasing their preference for that male, accordingly. MCC generalization occurs when males with similar phenotypes to the first one are also preferred. This behavioural pattern has been found in several species, including one species of invertebrate, *Drosophila melanogaster*. MCC may lead to reproductive isolation between populations from the same species, since it can be responsible for a cultural divergence of mating preferences. However, if immigrant individuals copy the choices of natives, this can also lead to hybridisation events. Moreover, since MCC changes female preferences based on male attractiveness, they might wish to invest more in the offspring of those attractive males (differential reproductive allocation). Taking all these into account, we were able to see that MCC is present in another invertebrate species, *Drosophila subobscura*. We simulated a scenario where populations from the two extremes of this species latitudinal cline (NL from the Netherlands and PT from Portugal) immigrated to a new (lab) environment with a resident TA population (adapted to the lab for several generations). Our aim was to test the hybridisation and differential allocation hypotheses of NL and PT with TA. We found that MCC is population dependent, occurring in PT but not in NL. PT females copied TA females when they chose PT males. However, copying seems to have little or no contribution to the process of hybridisation, because PT females' innate preference for TA males was already at 80% and copying TA females when they chose TA males did not increase this preference. MCC also seems to have no effect on female reproductive investment, although we found that female choice (compared to no-choice controls) can increase offspring's juvenile viability.

Key words: mate-choice copying; hybridisation; differential allocation; *Drosophila subobscura*; sexual selection

Resumo

Tendo como resultado o aumento do "fitness", conceito central em biologia evolutiva, as pressões selectivas podem ter levado os indivíduos a adoptar comportamentos no sentido de reduzirem o erro associado à escolha de um parceiro sexual.

"Mate-choice copying" (MCC) é a designação para a alteração da preferência sexual de uma fêmea (embora também possa estar presente em machos) mediante a informação social que recebe para um determinado macho, quando este acasala com sucesso com outra(s) fêmea(s). Pensa-se que o MCC permitem às fêmeas fazerem melhores escolhas, tendo assim um impacto positivo no seu fitness. O MCC pode ser generalizado a outros indivíduos com fenótipo semelhante, sendo este um componente essencial para a evolução de preferências culturais sexuais.

Este padrão comportamental tem vindo a ser descrito num número crescente de espécies. Contudo, em invertebrados, apenas se sabe estar presente em *Drosophila melanogaster*. Tem também vindo a ser implicado no reforço das barreiras reprodutivas entre espécies, ou mesmo populações da mesma espécie, podendo estar a contribuir para processos incipientes de especiação. Isto porque pode conduzir a preferências culturais divergentes dentro de uma mesma população, com indivíduos a adoptarem uma "cultura" e outros outra. Contudo, o mesmo se pode aplicar ao processo de hibridação – se um indivíduo imigrante passar a copiar os acasalamentos que observa no seio da nova população para onde imigrou, então poderá iniciar um processo de hibridação, facilitado pelo MCC.

O MCC também pode conduzir a alterações na forma como o investimento reprodutor é feito. Por norma, os indivíduos podem fazer um investimento reprodutor maior quando têm a possibilidade de acasalar com os indivíduos para os quais têm uma preferência sexual. Chama-se a este comportamento investimento reprodutor diferencial. Ora, se a informação social aumenta a atractividade dos machos e se o MCC tem a capacidade de aumentar a preferência das fêmeas por esses machos, então poderá também conduzir a um aumento do investimento reprodutor das fêmeas nas ninhadas produzidas com esses machos.

O presente trabalho foi, assim, composto por três objectivos principais: (1) descrição do MCC numa nova espécie de invertebrado, a *Drosophila subobscura*; (2) perceber o papel do MCC na hibridação; e (3) estudar o efeito do MCC no investimento reprodutor.

Drosophila subobscura é uma das espécies adequadas para a concretização destes objectivos, dado ser um organismo modelo para estudos de adaptação. Também são encontradas populações desta espécie altamente diferenciadas entre si, o que favorece os

estudos de hibridação. Estudos da equipa onde se desenvolveu este projecto têm estudado a adaptação ao laboratório de populações desta espécie, desde a sua introdução a partir de fundações na natureza até muitas gerações depois, e é neste paradigma laboratorial que se insere esta tese.

Em particular, foram fundados dois regimes no laboratório, provenientes dos extremos do cline Europeu desta espécie: PT (proveniente de Adraga, Portugal) e NL (proveniente de Groningen, Holanda). Durante este estudo, foi também utilizado um terceiro regime, TA, a servir de controlo, constituído por populações mantidas há muitas gerações no laboratório (e provenientes também de Adraga). Foram realizados dois ensaios de hibridação, em duas gerações distintas após fundação, as gerações 6 e 10.

O protocolo incluiu três fases: pré-demonstração, onde uma fêmea observadora PT podia escolher entre afiliar-se (sem contacto) a um macho PT ou TA; demonstração, onde duas fêmeas demonstradoras TA eram colocadas junto ao macho TA ou PT (transmissão de informação social positiva relativamente a esse macho); pós-demonstração, onde o comportamento de afiliação da fêmea observadora era de novo avaliado; e fase do acasalamento, onde era dada oportunidade à fêmea observadora de, efectivamente, escolher acasalar com um macho TA ou PT. O mesmo protocolo foi utilizado para as fêmeas observadoras NL. Este protocolo permitiu simular uma situação de chegada de indivíduos de uma população (NL ou PT) a um novo ambiente aonde já se encontrava uma população adaptada (TA).

O protocolo foi feito de forma a ser possível discernir uma preferência inata de uma preferência social, e ainda estas duas de uma escolha efectiva de acasalamento. Para testar a generalização do MCC, os machos usados durante a etapa da demonstração nunca foram os mesmos indivíduos usados depois para testar a preferência pós-demonstração, e para testar a escolha na fase do acasalamento. Contudo, na fase do acasalamento, não foi possível distinguir, efectivamente, a escolha das fêmeas da capacidade competitiva dos machos. Isto deveu-se ao facto de os machos utilizados terem vindo de regimes distintos e de terem, portanto, uma potencial capacidade competitiva diferente.

Por fim, foi permitido às fêmeas colocar ovos durante três dias, durante os quais os mesmos foram contados. De seguida foi estimada a viabilidade juvenil (% de ovos dando adultos) e estimado o rácio sexual, para analisar o efeito do MCC no investimento reprodutor das fêmeas. Paralelamente, foram mantidos casais controlo pré-definidos, não havendo uma escolha, por parte destas fêmeas, do macho com o qual iriam acasalar. Estes casais foram formados de forma a representarem todo o tipo de casais homogâmicos e heterogâmicos possíveis durante os ensaios.

Após a análise dos resultados, observou-se que a resposta à informação social está dependente do regime de onde provêm as fêmeas e também do grau de diferença fenotípica. Assim, apenas as fêmeas provenientes do regime PT fizeram MCC e apenas quando as diferenças fenotípicas entre este regime e o controlo começaram a ser menores – de facto, na primeira geração ensaiada, estas fêmeas não fizeram MCC. Em vez disso, evitaram os machos que viram a acasalar. Quando as diferenças se tornaram hipoteticamente menores, passaram a realizar MCC. Mas nesta fase, já a preferência inata das fêmeas PT pelos machos TA estava nos 80% (na primeira geração elas rejeitavam os machos TA) e, portanto, o MCC não aumentou essa preferência (o MCC foi apenas visível quando as fêmeas PT viam as TA escolher machos PT).

Já as fêmeas do regime NL, nunca responderam à informação social, mantendo a sua preferência inata por NL na primeira geração e por TA na segunda geração. Esta diferença entre PT e NL poderá dever-se ao facto de, na natureza, as fêmeas PT estarem adaptadas a uma elevada variabilidade no fitness dos machos, justificando o seu comportamento flexível, ao passo que a população NL sofre, regularmente, "bottlenecks", sendo a sua variabilidade genética menor.

Estes resultados obtiveram-se apenas nas fases em que não existia interacção directa com os machos, mas apenas visual (fases pré e pós-demonstração). Na fase do acasalamento, onde havia competição intrasexual, os machos da população controlo reproduziram-se significativamente mais devido à sua elevada performance competitiva, não havendo influência da informação social, ou, se houve, a interferência dos machos sobrepôs-se à escolha das fêmeas. Posteriormente, com a convergência de PT e NL para o ambiente de laboratório, a performance dos machos acabou por se tornar equivalente entre os do regime de controlo e os dos regimes introduzidos. Contudo, também nesta geração, a escolha das fêmeas na fase do acasalamento foi diferente da escolha na fase da pré e pós demonstração.

Em balanço, este estudo sugere-nos que o MCC, nas populações estudadas e nas condições experimentais testadas, parece pouco contribuir para a hibridação. De facto, quando a adaptação a novos ambientes leva a uma rápida evolução das preferências sexuais na população imigrante, se estas facilitarem a hibridação com a população local, o MCC pode assim tornar-se irrelevante. O MCC também não parece aumentar o investimento reprodutor – de facto, aquilo que faz aumentar o investimento é a presença de uma escolha face à ausência desta, mas não o MCC por si.

Palavras-chave: *Mate-choice copying*; hibridação; investimento reprodutor; *Drosophila subobscura*; selecção sexual

Index

Introduction

Sexual selection

Fitness is one of the most important concepts in evolutionary biology and is largely dependent on survival and reproduction of individuals. Both these factors are going to act on the possibility of individual's genetic contribution to the next generation and, therefore, to evolution. In the context of sexual selection, reproductive opportunities are, generally, scarce and individuals have to make choices to be able to maximize their own fitness.

Sexual selection was first described by Charles Darwin in 1871[1]. For him, some traits could not be explained by natural selection alone but only through a mate-choice or male-male competition scenarios. This evolutionary theory was opposed by Alfred Wallace, for whom sexual selection, in a situation that males fight over females, was another form of natural selection. He also denied the existence of a female choice, believing that natural selection was sufficient for the evolutionary explanation of almost all biological phenomena[2]. The debate between Darwin and Wallace created a controversy that led to the avoidance, by most evolutionary biologists, of research related to the subject of sexual selection that only ended with Fisher, who gave rise to the sexy sons' hypothesis[3].

Hypothesis for sexual selection under the genetic paradigm

Fisher, in 1930, tried to explain the "paradox of the lek"[3]. This paradox occurs when females seem to choose males with conspicuous phenotypes. These secondary sexual characters are so extreme that they reduce male survival. For him, disadvantageous phenotypes would have evolved if, by chance, females would have preferred this phenotype, which resulted in male and female progeny with the same characteristics and preferences, respectively. Theoretically, this originates a runaway process that gradually exaggerates the growing of the same physical peculiarity that was being chosen by the females in the first place, until natural and sexual selection balance each other[3]. Therefore, a male with large and/or brighter ornaments enjoys higher mating success, even with a cost to its own survival and viability, because that same characteristic is being preferred. This allows him to reproduce more and, consequently, to have more fitness. At the same time, a female gains an indirect genetic benefit by passing that same traits to her offspring that will have the same advantage. This form of indirect sexual selection

towards females leads, therefore, to an evolutionary exaggeration of a male trait that is genetically correlated to female preference that also becomes exaggerated[3].

The genetic paradigm of sexual selection was also present in alternative explanations to the same phenomenon, like the handicap principle[4]. In his hypothesis, Zahavi sees extravagant phenotypes of males as a proxy to male quality – low quality males cannot afford the costs of developing conspicuous secondary sexual characters; high quality males can. Therefore, conspicuous phenotypes serve as a costly signal available to females[4]. Since it is costly, it cannot be falsified and thus become a reliable source of information from males to females (and even to other males). In this way, females seemingly choose good genes for their offspring. However, two assumptions have to be made: condition dependence of sexually selected traits and high genetic variance in condition[5]. Both of them have now extensive empirical support[6–8].

Another hypothesis[9] suggests the same, but in the particular case of host-parasite arms race – males with brighter display are correlated with higher resistance to parasites. It creates a cycle that reinsures a continual source of genetic and fitness variation that solves the necessity of variance in life-history traits, due to the continuous adaptation of the parasite to the host and vice-versa.

These are the main hypotheses for sexual selection and all of them assume that male traits are heritable and that they are inherited together with the female preference for those traits, implying that a genetic linkage needs to be present[10]. Therefore, under these assumptions, female mate preferences are independent from any kind of social experience and are constant through life.

Hence, until a certain period, most sexual selection studies have been made assuming that a female's preference is its genetic preference, not taking into account the potential effect that the social environment can have on the ontogeny of an individual's choices. But, unless the conditions are stable, inherited genetic information in how to behave and what choices to make may be useless – because those adaptations could have evolved for other abiotic or biotic environmental conditions than the ones that the animal is currently experiencing. Therefore, individuals need to gather information about their environment, either through trial-and-error or through interactions with conspecifics – personal information and social information, respectively. This last one can be based on signals (i.e. intentional communication) or on cues, if provided inadvertently by the performance of individuals in their daily activities (inadvertent social information)[11]. However, learned behaviours and preferences cannot have any impact on evolution (and consequently on sexual selection) if it they are not inherited. The classic concept of heritability says, however, that the only component that directly passes down from generation to generation is the additive genetic variation[3]. Although the concept of additive

genetic variation have been widespread in evolutionary biology, the broad use of social information by animals in a lot of different contexts[12–15] calls for a revision in what is considered to be inherited.

Danchin proposed a new concept of heritability[16] that take this into account. For him, there are two main components that contribute to phenotypic variance: the transmitted component and the non-transmitted component.

The transmitted component includes the classic genetic component of transmitted variance, but also the non-genetic transmitted variance, which includes epigenetic variance[17], parental effect variance[18], habitat inheritance and social variance (also named culture)[19]. Therefore, a concept of heritability should encompass all that is inherited across generations, which Danchin call "inclusive heritability".

If we take this into account, then we don't need to always evoke a genetic linkage between a sexual trait and the preference for that same phenotype, because female sexual preferences and even male sexual displays can be maintained through culture or social learning.

Cultural hypothesis and mate-choice copying

One category of social learning that can have a huge impact on sexual selection is mate-choice copying (MCC). Defined by Wade & Pruett-Jones[20] and first observed by Dugatkin[21], MCC occurs when an observer female (although MCC can also be present in males it is most commonly studied on females) receives information about the performance of a male, increasing or decreasing her preference for that male, accordingly. For Danchin[11], this happens in a context of inadvertent social information.

MCC can be advantageous, since it can reduce the uncertainty that comes with a sexual choice. However, it can also have a conservative role on the evolution of male traits in a scenario with a predictable and homogeneous environment[22]. Although MCC may not be adaptive in this scenario, it could be under heterogeneous and unpredictable environments. This is extremely important because the phenotypic value of a male is largely dependent of the ecologic context and, therefore, genetic preferences may not mirror the quality of a potential sexual partner in a given place and in a given moment in time. Having background information about the sexual preferences of other individuals, particularly if they are older and, therefore, more experienced[23], can increase the certainty of the quality of a sexual partner, since it can be expected that individuals being copied already assessed the individuals that they are choosing. Also, since it happens in an inadvertent social information context, it can't be falsified, being a trustworthy cue that the observer can use in its own sexual choices.

Social generalization can also occur as a by-product of MCC, when a particular trait is being copied and not the individual[24]. This can give rise to the cultural inheritance of preferences and fix different sexual preferences in different populations of the same species, potentially starting a process of pre-copulatory reproductive isolation.

MCC have been found in several species[25]. For instance, in Galef's study[26] they show that Japanese quail females spend more time with a less-preferred male only if, previously, they saw another female spending time with that male. Social generalization also occurs[27], as the observer females increased their preference for every male with the same phenotype.

These evidences support the idea that social learning during mate choice can be seen as an important mechanism of sexual selection and even, in our opinion, an alternative mechanism to both Fisher's and Zahavi's hypotheses: when an observer female copy the choice of another female, she does not know if the trait being chosen was carefully assessed or not; if it was, the cultural inheritance of the preference for that trait can give rise to an evolutionary process similar to the handicap principle; but if the trait was randomly chosen, its cultural inheritance can give rise to Fisher's runaway process. The cultural inheritance of preferences can happen or be potentiated via a process of informational cascade, which occurs when the acquired information about male quality by a female is based on the behavioural decisions of other females and not on the male cues on which the decisions from the first females were based[28]. Hypothetically, this process has the power to fix a choice or behaviour on a population. Even if there is evidence of a genetic preference – and in several species evidence has been found[29,30] –, it doesn't mean that it has started that way, since a cultural preference can be established first and the genetic preference only evolve secondarily. This evolution from cultural to genetic preference would be more likely when social learning is costly – in some species there may be a trade-off between learning abilities and fertility or survival[31] – and/or when the environment becomes more predictable or homogeneous. Indeed, MCC may be particularly advantageous if the environment is heterogeneous, as explained above. In this situation, it is unlikely that genetic preferences and behaviours are adapted to every possible situation to increase individual fitness. Therefore, they need to have some behavioural plasticity to overcome this limitation – this could be accomplished through social learning.

This is a cultural hypothesis for sexual selection. Evidences already exist for the social learning and social generalization of female mate preferences. The rest of it is hard to test, but efforts should be made in this direction since, if it is right, it could change the way we see evolution and sexual selection.

Mate-choice copying in invertebrates

MCC has been found in different species of vertebrates[25]. On the contrary, there's a lack of empirical studies of MCC in invertebrates. Mery & Varela[32] were the first to find evidences of MCC (and its social generalization) in an invertebrate species, *Drosophila melanogaster*. Using two different protocols, they were able to show that females can change their genetic preference when in presence of social information. In experiment 1, observer females could choose to spend more time with good-condition or poor-condition males, without any direct contact (males were enclosed in a petri-dish). Although they preferred the good-condition males, their preference increased for the poor-condition males after receiving positive social information about them (model females were enclosed together with these males) – this provided evidence that MCC is present in this species. In experiment 2, two artificial phenotypes were created – pink and green males – both in good phenotypic condition. Half of the observer females received positive information for pink males and negative information for green males and the other half received the opposite treatment.

In this second experiment, the positive information was given by enclosing males with virgin females (responsive to courtship and so to copulation) and negative information by enclosing males with non-virgin females (non-responsive to courtship nor to copulation). After six trials of learning (three positive trials for a phenotype and three negative trials for the other one), females were able to choose between two other males of the same pink and green phenotypes. These males were not the same individuals that were used in the learning trials; therefore, females could only copy the phenotype and not the individuals themselves. Mery & Varela verified that *Drosophila melanogaster* was able not only to do MCC, but also social generalization of that preference, this last one being essential for a process of cultural inheritance to happen.

Surprisingly, in the same year, Auld[33] didn't find evidences for MCC in *Drosophila serrata* – there weren't any female bias toward males that were preferred by other females. They used a protocol very similar to the second experiment of Mery & Varela[32].

To our knowledge, these are the only studies that tried to find MCC in invertebrates. Therefore, we don't' know if this behaviour is specific of *Drosophila melanogaster* or if it is widespread in other species of invertebrates and, specifically, other species of *Drosophila*. Theoretically, species that have a large refractory period after a first successful mating should have a stronger tendency to MCC behaviour, since mating opportunities for females are scarce[22]. *Drosophila melanogaster* females can remate, but much less often than *Drosophila serrata* that

have almost no remating latency, probably due to a strong post-copulatory sperm competition process[34].

Females from *Drosophila melanogaster* can also avoid males that they saw previously mating using both chemical and visual social information[35]. However, this species seems to be able to discriminate between two different individuals of the same populations and, consequently, doesn't contradict previous data obtained by Mery & Varela[32]. Loyau[35] explain their findings by a female avoidance of males with sperm depletion. 24 hours later, females no longer avoid the individual males that they had seen mating before. Still, this could imply that MCC may not occur in species where males become sperm depleted after the first copulation or only occur after a while, when males are already with their basal amount of sperm. In the experiment 1 of Mery & Varela[32], MCC was tested one day after the learning trials and, therefore, these two studies are not contradictory, since enough time was given for the males to replenish their basal level of sperm. This did not affect, however, the social generalization of the preference acquired during MCC (experiment 2), since it was the phenotype that was being copied, not the male.

One of the objectives of the present study was to investigate if MCC is present in another *Drosophila* species, *Drosophila subobscura*. Under the previous hypothesis, we expected that this species has a strong MCC behaviour, because its mating latency is even larger than that of *Drosophila melanogaster*[36]. However, there could be other factors limiting this type of social learning in *Drosophila subobscura*. Also, this is a model species in highly replicated and controlled, real-time evolution studies of adaptation to new environmental conditions[37], a central issue for the questions that we are addressing (see further below).

Mate-choice copying: speciation and hybridisation

Assortative mating has been always seen as a way to reinforce a prezygotic reproductive isolation[38] and its normally considered to be a genetic determined feature of the individuals. But, according to the MCC theory, the same can be achieved through social learning, because individuals can learn with their conspecifics which mate choices should they make, with consequences to both the fitness of the copying and of the copied individuals. This can lead to the isolation of populations with different "traditions" of mating preferences and, consequently, to evolutionary divergence and posterior speciation. We don't need to evoke an allopatric scenario, because different social groups can be formed in sympatry, creating "social islands" – a concept expanded from sympatric speciation theory that affirms «if the population inhabits two subenvironments or "niches", the population size being separately regulated to numbers

N1 and N2 in the two niches, and if AA is fitter in one niche and aa in the other, then a stable polymorphism is possible»[39]. In specific conditions (like assortative mating and habitat selection, for instance), this could result in speciation and the formation of two different species. Giving enough time, this can lead to an increase of phenotypic differences that reinforces the process of divergence in a way that hybrids become less and less viable.

Cultural differences may appear after the process of divergence had already started, or be at the origin of the process. Indeed, it would be important to test in the future which event, genetic or cultural divergence, is more likely to have had a direct causality in speciation. Several theoretical and empirical studies have been focusing on the potentialities of cultural evolution on the evolutionary history of populations[40,41].

Similar to genetic evolution, cultural evolution can be affected by copying errors or improvisation (similar to genetic drift and mutation), by cultural selection (similar to natural selection) and by "meme flow" (similar to migration)[11]. All of this can change the cultural background of the populations and, therefore, restrict social interactions between individuals of the same species – as it happens in the human species, individuals are more likely to interact with individuals that share the same culture than with others that don't. Being part of the concept of culture, MCC can also act as a potential mechanism of divergence through cultural evolution and, therefore, could also guide and/or contribute to a process of speciation.

The opposite scenario is also possible. MCC followed by social generalization, can also break the process of differentiation through hybridisation, because the learning of social and cultural preferences from a different population allow individuals to hybridise, even if some phenotypic differences are already present. The main objective of our work was precisely to investigate if individuals from different populations can learn with one another and start a process of hybridisation through MCC in *Drosophila subobscura*. This could be particularly advantageous if one population is arriving to a new environment with different ecological characteristics. Therefore, by reproducing with local individuals already adapted to the new environment, they can increase their own fitness. The advantage of hybridisation is significant, at least when the hybrids are better fit than the parental phenotypes. It can increase genetic (and cultural) variation of the mixed population and consequently accelerate their adaptation to novel or changing environments[42].

The foundation of new regimes is, therefore, needed to simulate the arrival of new individuals to a new environment. Here we use the laboratory environment as the new environment and long-established populations of *Drosophila subobscura* as the local populations, while the invaders will be of two sources, in order to analyse the role of population differentiation on the outcomes. *Drosophila subobscura* is a particularly interesting species to

analyse the role of social learning in hybridisation. This species has a clear latitudinal cline in Europe, both in body size and chromosomal inversions, repeatable after colonization of the New World[43]. Previous studies of laboratory adaptation involving populations along the latitudinal cline in Europe showed clear initial differentiation followed by quick convergence for several life-history, physiological and morphological traits[37].

It becomes thus of high interest to analyse populations derived from contrasting latitudes for social learning per se as well as its possible role in hybridisation, as is proposed here. Also, some species of *Drosophila* show clinal variation in sperm length[44], reinforcing the importance to use different populations of the latitudinal cline to account for possible differences in sexual and mate-choice behaviour.

Mate-choice copying and differential allocation

Nancy Burley, in 1986[45], proposed the hypothesis of differential allocation, where she argued that an individual's attractiveness affects the parental investment of its mate. Parental investment in the offspring should, thus, be higher when a female sexual partner is more attractive and should be smaller when less attractive. Higher investment could lead to more offspring and/or more competitive individuals.

This is particularly advantageous to the females due to their higher investment in reproduction and, consequently, parental care – a female will trade-off future reproduction in the presence of an attractive male, increasing their own reproductive investment indirectly through parental care or directly through a larger brood or larger eggs[46]. Burley confirmed her own hypothesis[46]. Additionally, the sex-ratio of the offspring can also change in response to the attractiveness of the male, as proposed by Trivers & Willard[47] and supported by empirical evidences[48]. Individuals will produce more offspring of the most attractive sex within the breeding pair.

When present, MCC can change the attractiveness of individuals. Therefore, it has the potential to also change the way parental investment and sex-ratio of offspring is going to be carried out. In this study, our last objective was to study if fecundity, juvenile viability, and sex-ratio is influenced by the change in the attractiveness of the males due to MCC. Will fecundity, juvenile viability, and the number of males of the produced offspring increase when the attractiveness of the male is socially increased by MCC?

Objectives

Putting all together, the big question of our work was to study whether MCC can lead to the hybridisation of previously geographically isolated populations. At the same time, this allowed us to test if MCC is present in a new species of invertebrate, *Drosophila subobscura*, and to see if MCC can have an important impact in the reproductive investment of the females. Consequently, there were three main questions that we tried to answer: Is MCC present in *Drosophila subobscura*? Can MCC lead to hybridisation? And does MCC increase reproductive investment?

Material and methods

Foundation and maintenance of populations

Drosophila subobscura individuals were collected in August 30th and 31st of 2013 from Adraga, Portugal, and between August 30th and September 1st of 2013 from Groningen, Netherlands. These were used to give rise to new sets of laboratory populations (from here on called 'regimes', as opposed to populations, a term that will be reserved for the three replicate populations; see below). A first triage for *Drosophila subobscura* was done in the field and a more precise one was made in the laboratory using specific phenotypic markers of this species[49]. The number of founding females was 192 for Adraga (PT) and 138 for Groningen (NL). Females were maintained in separate vials during the first two generations, to ensure a similar contribution of the founders to the next generation. F1 eggs and the individuals themselves were treated with tetracycline (25 mg/l) and F2 eggs with ceftriaxone and spectinomycin (50 mg/l) to avoid contaminations due to suspected presence of pathogenic bacteria that would lead to high larval mortality. Tests in previous foundations from the same locations ensured that no *Wolbachia* was present in these founders or other regimes already present in the laboratory[37].

To avoid inbreeding, F1 females were crossed with males from different vials, and females from F2 were crossed with males from a random sample of vials. Finally, at the 3rd generation, an equal number of offspring of each female were randomly mixed, giving rise to the outbred regimes. At the 4th generation, each regime (PT and NL) was split into 3 replicate populations by dividing the eggs laid by F3 females into three different populations (originating NL_1, NL_2 and NL_3 for the NL regime and PT_1, PT_2 and PT_3 for the PT regime). Another regime (TA) was used as control also with three replicate populations (TA_1, TA_2 and TA_3). This one was founded in 2001 from individuals collected in Adraga, Portugal, and was at its 153th generation at the time of founding of the newly introduced populations. This regime was also treated with the same antibiotics to ensure uniform conditions across regimes. An assay done in a past study indicated no significant interaction between antibiotic treatment and the different foundations[37].

The three regimes were maintained under the same standard conditions, following the long-term maintenance procedures of the Matos lab[37]. These include: discrete and synchronous generations of 28 days; reproduction close to peak fecundity, with egg collection for the next generation from females between 8 and 12 days of age after emergence; eggs kept in vials with

controlled density (70 eggs per vial); temperature at 18ºC, except during manipulation, and photoperiod of 12h L: 12h D; random mixing using CO_2 anaesthesia of imagos from each replicate population, emergent during the first 4 or 5 days, assigned to several vials (50 adults per vial). Census sizes between generations 4 and 11 were between 520 and 1300, with an average of 1032, 1081 and 1111 for PT_{1-3}, NL_{1-3} and TA_{1-3}, respectively.

Assays

Two assays were planned during this project. The first assay at the 6th generation and the second assay at the 10th generation.

To obtain the individuals for the assays, in the corresponding generation an additional egg collection was made. This occurred during the first three days (each day for a different replicate population of each regime, e.g. NL_1, PT_1 and TA_1 on day 1) after the egg collection for maintenance of the populations. Adult flies were collected from the second and third day of emergence. Sex screening was made shortly after the collection of the imagos to ensure that all individuals were virgin, using CO_2 anaesthesia. Four days before the trials, males were anaesthetised again with CO_2 to be painted with the specific colours that would be used in the assays. This allowed them to recover from the negative effects of the anaesthesia in their sexual behaviour[50].

Since the three replicate populations from each regime had its eggs collected in different days, each day involving one population from each regime, this allowed to do the assays in three different days, using individuals with approximately the same age across populations and regimes.

The objective of the assay was to study MCC in a new species of invertebrate (*Drosophila subobscura*) and to try to understand the role that MCC can have on the hybridisation of individuals of different populations from the same species. This assay was done in four steps: 1) Pre-demonstration step; 2) demonstration step; 3) post-demonstration step; 4) mating step (figure 1). The males that were used in all of these steps were either painted, on their dorsal thorax, with a dot of red nail polish or black nail polish. Since species of *Drosophila* are not able to see the red colour[51], this allowed the researchers to distinguish between different males, while females could only use the natural phenotype of the males to distinguish between them. The black colour mixes up with the natural blackish colour of the *Drosophila subobscura* species, and was used as a control.

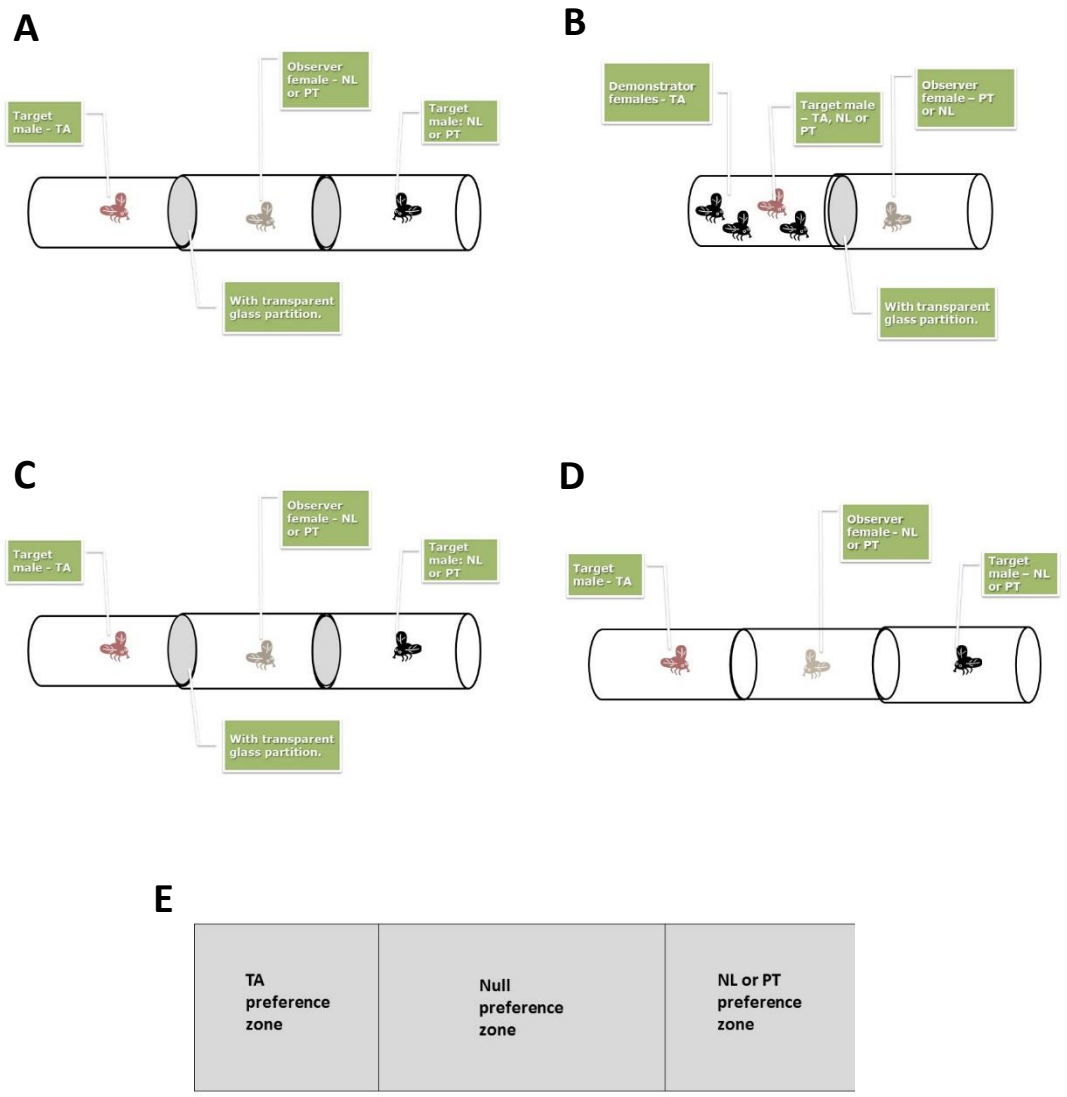

Figure 1. Different steps of the assay. This protocol tries to study if MCC can increase the hybridisation between individuals from different populations, with four different steps: (A) pre-demonstration step; (B) demonstration step; (C) post-demonstration step; (D) mating step. The female is in the central tube, where three preference zones were identified (E): TA preference zone; null preference zone; and NL and PT preference zone.

In the pre-demonstration step (figure 1A), the innate preference of the females was measured. For that, a tube with two opened ends was used. Each end was in contact with another tube, separated by a transparent glass partition (a microscope cover glass). These two additional tubes were then used to give different stimuli to the observer female placed at the central tube, generating three 'preference zones': TA preference, null preference and NL/PT preference (figure 1E). The TA preference zone was in contact with the tube where a male from the control regime was present; the NL or PT preference zone was in contact with the other tube, where a male from the observer female's regime (NL or PT) was present. Finally the null preference zone was the area between the TA preference zone and the NL/PT preference zone. The observer female was allowed to move between the different preference zones. In such a

set-up, females only observe males from TA or from their own regime. This allows to measure the time that each female spent in each preference zone and, by consequence, to measure their innate preference.

In the demonstration step (figure 1B), the same observer females from the previous step were used. However, now there were no preference zones – the female could only see one male with two other, virgin females – the demonstrator females, both from the control regime. For half of the observer females, the male she observed was from her own population, while, for the other half, the male was from the control population. Since the demonstrator females were virgin, they would copulate with the males, giving positive social information about that male to the observer female. Therefore, half of the NL females received positive information about the TA males and the other half about the NL males. The same happened to the PT females: half of them received positive information about the TA males and the other half about the PT males. Consequently, there are two treatments for each of the two regimes used. The first scenario simulated a situation in which a female arrives to a new population and sees the local females choosing mating partners from that same population, while the second scenario serves as a control, possibly reinforcing innate preference.

In the post-demonstration step (figure 1C), the objective was to see if the demonstration had any influence on the preference of the female. This step was similar to the pre-demonstration step, with the three preference zones.

Finally, after the post-demonstration step, the transparent glass partitions that were keeping the individuals separated from each other were removed to allow the female to choose a male to mate (figure 1D). Each one of these three four steps lasted one hour and a half.

After copulation, the females were transferred to a tube with medium to allow them to lay eggs. The females were transferred daily during three days to a new vial with fresh medium and the number of eggs laid per female counted. These eggs were then stored at normal maintenance conditions to allow them to develop. After development, the number of imagos emerging from the vials were counted and the sex-ratio calculated.

Heterogamic (NL(F)-TA(M) and PT(F)-TA(M)) and homogamic (NL-NL and PT-PT) couples (without receiving any kind of learning or chance to choose) were also made as controls to see if social information could have an impact on the reproductive investment of the individuals. As before, the individuals were transferred daily during three days and the eggs were also stored to allow for their development.

The pre-demonstration and post-demonstration steps were both tape recorded to measure the number of times that the female spent in each preference zone. Nine sampling points, separated by 5 minute intervals (after the first twenty initial minutes), were used to

estimate the preference of the female. In each of these nine points, we saw where the focal females were and noted down accordingly. The post-demonstration step, before the removal of the transparent glass partitions, was necessary because males from the control regime have a better performance since they are already adapted to the laboratory conditions. Without the comparison of the pre-demonstration step with the post-demonstration step and the mating step we would not be able to discriminate between female preference, after demonstration, and male competition.

We considered that a female had a preference for a particular male if she was in his preference zone for more than a third of the total number of points and if it was higher than the proportion of points that she had spent with the other male. If she would have spent more time in the null preference zone, we considered that she had no preference. 30 observer females per treatment (two treatments) were used for each of the three replicate populations from each regime (PT and NL). The total of the sample was 360 observer females. This assay was done at the 6^{th} and 10^{th} generations of NL and PT (and corresponding 159^{th} and 163^{rd} generations of TA).

Statistical Methods

A generalized linear mixed model fit by maximum likelihood was used at each of the assays to test for differences between populations in their response to the social information that they received regarding mate-choice decisions. The analysed differences between populations were behavioural (whether assortative preference or mating occurred or not after demonstration step) and fitness related (whether differential reproductive allocation occurred after MCC).

The following model was used to analyse specifically female preferences for males of their own population (assortative preference) in the pre-demonstration step:

$$Y = Reg + Gen + Pop\{Reg\} \quad (1)$$

Y refers to the trait being analysed (assortative preference, as mentioned above), with two categories: yes (choice of NL or PT) and no (choice of TA). *Reg* refers to the regime (NL or PT); *Gen* refers to the generation of the assay (6^{th} and 10^{th}); and *Pop{Reg}* is the random factor with the replicate population nested in regime. *Reg* and *Gen* are both fixed factors and were analysed with and without interactions. If any interaction or fixed factor was considered non-significant, there were subsequently removed with a backward stepwise procedure. This procedure was continuously applied until the best reduced model was obtained. For this analysis, the model fit was made with a binomial distribution, which is a discrete probability

distribution of the number of occurrences between only two possible options: yes or no for assortative preference. The aim of this analysis was to see if there is, in NL and PT females, an innate (genetic) preference for the males of their own population when compared to TA males. Assortative preference is expected (H1), at least, in the 6th generation. With convergent adaptation of PT and NL flies to laboratorial conditions, assortative preference in generation 10th might decrease.

The second model was used to analyse female preferences in the post-demonstration step and in the copulation step:

$$Y = Reg + Gen + Treat + Pop\{Reg\} \quad (2)$$

Y refers to the trait that was analysed: female assortative preference in the post-demonstration step and assortative mating in the mating step, both with two categories: yes (choice of NL or PT) and no (choice of TA); *Reg* refers to the regime (NL or PT); *Gen* refers to the generation of the assay (6th or 10th); *Treat* refers to the treatment that the observer females had received, with two categories: +, if they received positive social information about the male from their own population, and 0, if they received positive social information about the male from the control regime; and *Pop{Reg}* is the random factor with the replicate population nested in the regime. *Reg*, *Gen* and *Treat* were analysed with and without all possible interactions and all of them were fixed factors. Non-significant interactions and fixed factors were removed with a backward stepwise procedure, until the best reduced model was obtained. Like in the previous analysis, the model fit was made with a binomial distribution, since the response variable has only two possible options: yes or no for assortative preference and mating. This model was used to see if MCC is present in the two different regimes and how it evolves between generations. If it is present, the prediction (H1) is that assortative preference and mating differs in favour of the male for which the female received positive information.

A third model was also used to test for MCC, this time testing differences of female assortative preference before and after the demonstration step within each generation:

$$Y = Reg + Treat + Difer + Pop\{Reg\} + Focal \quad (3)$$

Difer discriminate if the preference was before or after the female had received positive social information and *Focal* is used due to the pseudo-replication effect. As before, all possible interactions were analysed. The prediction (H1) is that assortative preference should decrease when the observer females received positive information about the TA males. When females received positive information about the males of their own population (NL or PT), no behavioural change is expected if their preference was already for the males of their own population.

A generalized linear mixed model fit by maximum likelihood was used as well to analyse female reproductive investment after MCC. The following fitness related traits were analysed as response variables: number of eggs laid by the females (multinomial variable; requires a model fit with a poisson distribution), number of eggs that developed into adults (also multinomial), egg-to-adult juvenile viability ratio (binomial variable; requires a model fit with a binomial distribution) and adult sex-ratio (percentage of individuals that were male; also binomial). For each of the response variables the following model was used:

$$Y = MCC + Copulation + Reg + Gen + Pop\{Reg\} + Focal \quad (4)$$

Y refers to the traits that were analysed (referred above); *MCC* refers to the occurrence/non-occurrence of MCC (with three categories: yes, no or control); *Copulation* to the male that the female had copulated with (NL/PT or TA); *Reg* refers to the regime (NL or PT); *Gen* refers to the generation of the assay (6^{th} or 10^{th}); *Pop{Reg}* is the random factor with the replicate population nested in the regime and *Focal* is the random factor for the observer females that is used to control for over-dispersion (this is the technique used in generalized linear mixed models, where the model fit is made with binomial and poisson distributions). A poisson model was used to analyse the number of eggs laid and the number of adults and a binomial model was used to analyse juvenile viability and sex-ratio. *MCC, Copulation, Reg and Gen* were analysed with and without all possible interactions. Interactions and factors were removed if not significant with a backward stepwise procedure, which was used to reach the best reduced model. This model was used to study if MCC was changing the reproductive investment of the females. According to the differential allocation hypothesis, an increase in

female investment is expected after MCC (H1): more eggs, more adults, a larger juvenile viability ratio and a larger proportion of males.

All the tests that involved multiple comparisons were also corrected using FDR correction[52]. All of these analyses were made using R[53] with the lme4[54] package and using Excel 2013.

Results

This assay was done in two different generations, as mentioned above. At the 6th and 10th generation, female assortative preference for males from their own population was analysed before and after the demonstration step. After the mating step, the variables analysed were females assortative mating and female differential reproductive allocation, which was tested for different fitness related traits (number of eggs, number of adults, juvenile viability and sex-ratio).

The best reduced models for post-demonstration assortative preference included interactions with all fixed factors (regime, generation and treatment). For assortative mating and pre-demonstration assortative preference, the fixed factors present were regime and generation in interaction. None of the models included the random factor of the population nested in regime. In the mating step, the final choice was described as being a mating preference of the female – however, this was a simplification in order to avoid confusion. Indeed, the mating choice can also have a male component that cannot be discarded.

The best reduced model for the analysis of fitness related traits included the random factors of the population nested in regime and the focal female, except for sex-ratio, where population nested in regime was non-significant. Regarding the fixed factors, the best reduced models included *Copulation* and the covariate *Gen* in interaction. For the total number of adults and juvenile viability, the fixed factor *MCC* was also present in interaction with *Gen* as an alternative reduced model.

The results are presented first with the analyses of traits within each generation. After the results of the 10th generation, a comparison is made between the two generations. Tables with full static results about the models are given in the appendix.

Generation 6

Assortative preference was found for females from both regimes (NL and PT), that is, they had an innate preference for the males from their own population ($p<0.001$ for both of them). No differences were found between regimes in the degree of preference ($p=0.46$), which was 76% for NL and 80% for PT (figure 2A; table S1).

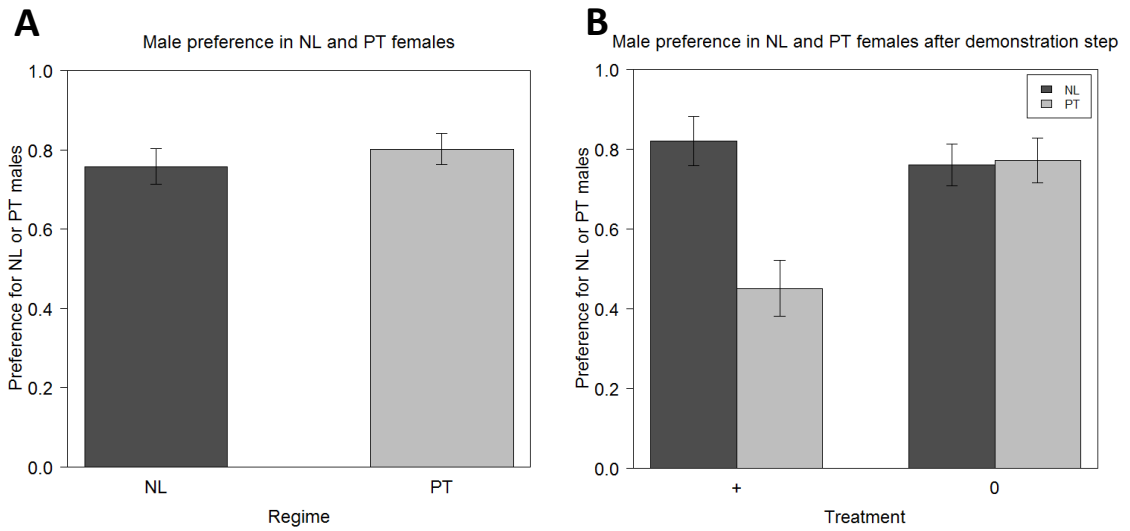

Figure 2. Female assortative preference for NL or PT males in the 6th generation. The preference of the NL and PT females was analysed before (A) and after (B) the demonstration step, in which females received positive social information about one type of male: + for the males from the females' own population and 0 for the males from the control population (TA). Error bars represent the standard error of the mean defined with the difference between individuals.

After receiving positive social information about one of the males, the females from the two regimes responded differently to the treatment (figure 2B; table S2). NL females did not show differences between treatments ($p=0.48$), having maintained their preference for the males from their own population ($p<0.001$ for both treatments). Therefore, no differences were found before and after treatment in what NL is concerned ($p=0.47$ for + treatment and $p=0.67$ for 0 treatment; table S3). The same did not happen in the PT regime. There is an effect of the positive social information: there are differences between treatments ($p<0.001$), with PT females showing no preference for either male in the + treatment (50% of assortative preference; $p=0.48$), but showing a preference for males from their own regime in the 0 treatment ($p<0.001$); the preference in this last treatment is no different from the preference before treatment ($p=0.99$; table S3), but the same does not happen with the + treatment, where there is a decrease in assortative preference ($p<0.001$; table S3).

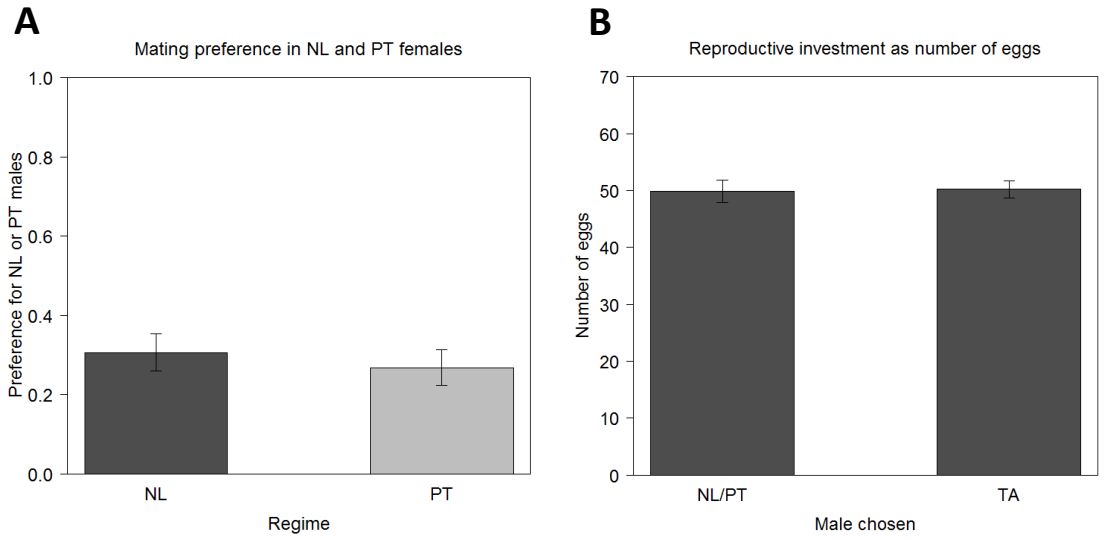

Figure 3. Female assortative mating and number of eggs laid in the 6th generation. The preference of the NL and PT females was also analysed in terms of mating choices, after the post-demonstration step (A). The number of eggs laid by those same females was counted during three days and is used as a proxy of reproductive investment (B). This variable was analysed as a function of the male chosen in the mating step (NL/PT or TA). Error bars represent the standard error of the mean defined with the difference between individuals (A) and between populations (B).

In the mating step, TA males performed better than males NL and PT, being responsible for 73% and 69% of the copulation with females from PT and NL regimes, respectively (figure 3A; table S4; $p<0.001$ for both regimes). There were no differences between regimes in mating preference ($p=0.56$) and it does not seem to be present any influence of social information, since the treatment variable was non-significant ($p=0.5128$).

Concerning the reproductive investment in terms of total number of eggs, no influence of the variable MCC was found ($p=0.4315$). The reproductive investment with TA males is similar to the investment with NL/PT males, with the variable *Copulation* being non-significant ($p=0.712$; figure 3B; table S5). The number of eggs laid is also not different between regimes ($p=0.713$).

After the development of the eggs, we analysed the total number of adults that emerged (figures 4A and 4B; table S6 and S7), juvenile viability from egg to adult (figure 4C and 4D; table S8 and S9) and sex-ratio (figure 4E; table S10). We found that the total number of adults and juvenile viability are dependent only on the male chosen during the mating step (*Copulation* variable), with these two traits being higher in females that copulated with TA males than in females that copulated with NL/PT males ($p=0.00176$ for total number of adults and $p<0.001$ for viability; figure 4A and 4C; table S6 and S8). There were no significant differences between regimes for total number of adults ($p=0.1596$) and for viability ($p=0.5003$). The regime also did not play a role in sex-ratio ($p=0.244$), nor the males chosen by females in the mating step ($p=0.279$). However, the variable sex-ratio is biased towards females, being approximately 56% for both regimes ($p<0.001$ for both of them; figure 4E; table S10).

The variable *MCC* did not seem to have influenced female reproductive investment and was not present in the best reduced models of generation 6: both females that copulated with the male for whom they received social information and females that did not are no different from the control, both in the total number of adults ($p=0.284$ and $p=0.211$, respectively) and in juvenile viability ($p=0.113$ and $p=0.889$, respectively; figure 4B and 4D; table S7 and S9). Differences were also not found between them ($p=0.044$ – but not significant when FDR corrected – for total number of adults and $p=0.131$ for juvenile viability). MCC also does not play a role in sex-ratio ($p=0.076$). These results are shown here for comparative reasons with generation 10, where MCC becomes relevant for total number of adults and juvenile viability (see below).

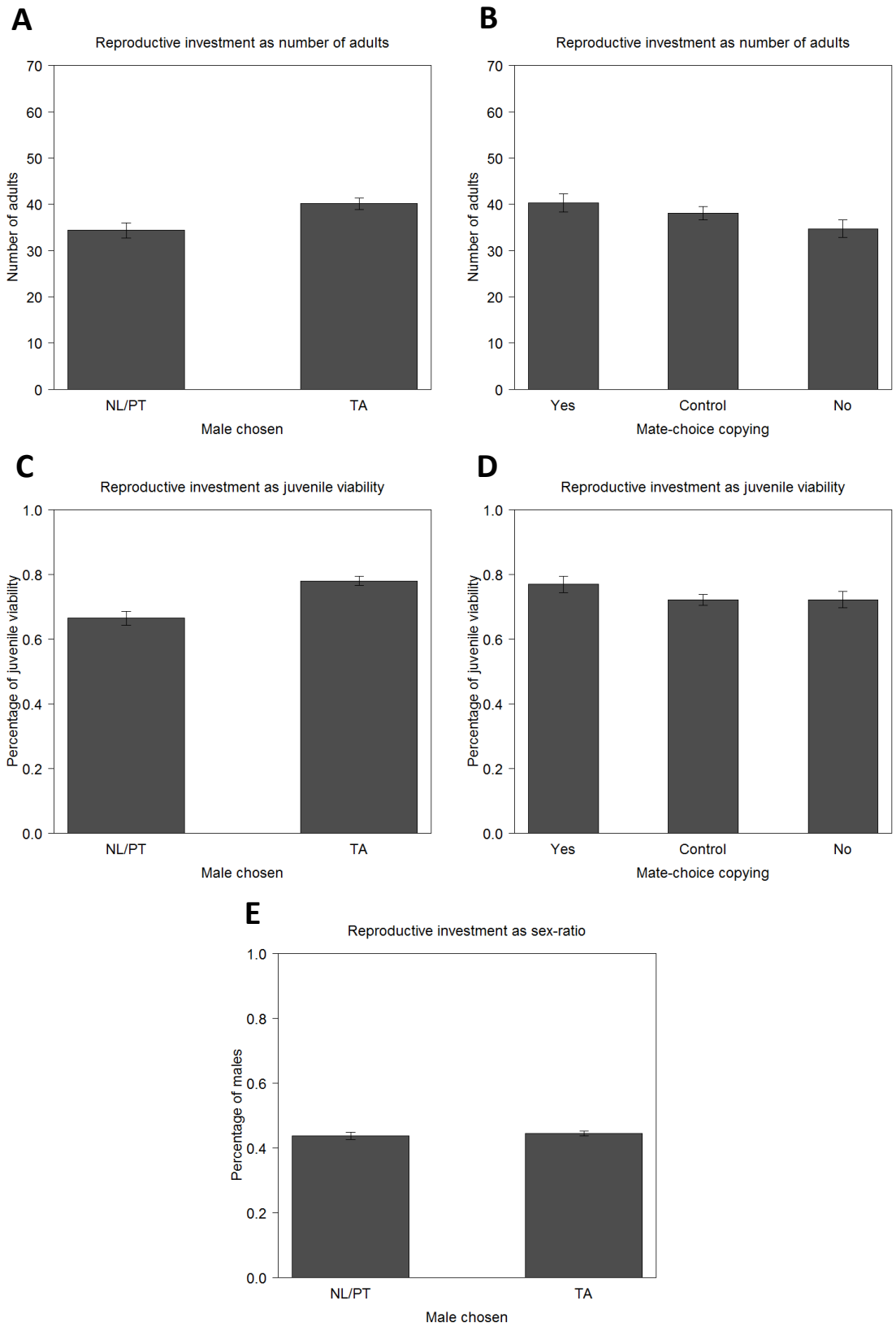

Figure 4. Traits related with female reproductive investment and mate choice in the 6th generation. The number of adults that emerged from the eggs laid during three days by the observer females (A and B), their juvenile viability (C and D) and their sex-ratio defined as percentage of males (E) in relation to the male chosen during mating step (A, C and E) and in relation to the occurrence/non-occurrence of MCC (B and D). Error bars represent the standard error of the mean defined with the difference between populations (A-D) and between individuals (E).

Generation 10

At the 10th generation, the results were different from those described above. Females from both regimes preferred males from the control regime ($p<0.001$) and no difference was found between them ($p=0.188$), with NL having a percentage of preference towards TA of 73% and PT of 81% (figure 5A; table S1). This change in preference is statistically significant for both regimes when compared with the results obtained in the 6th generation ($p<0.001$ for both of them; figure 2A and 5A; table S1).

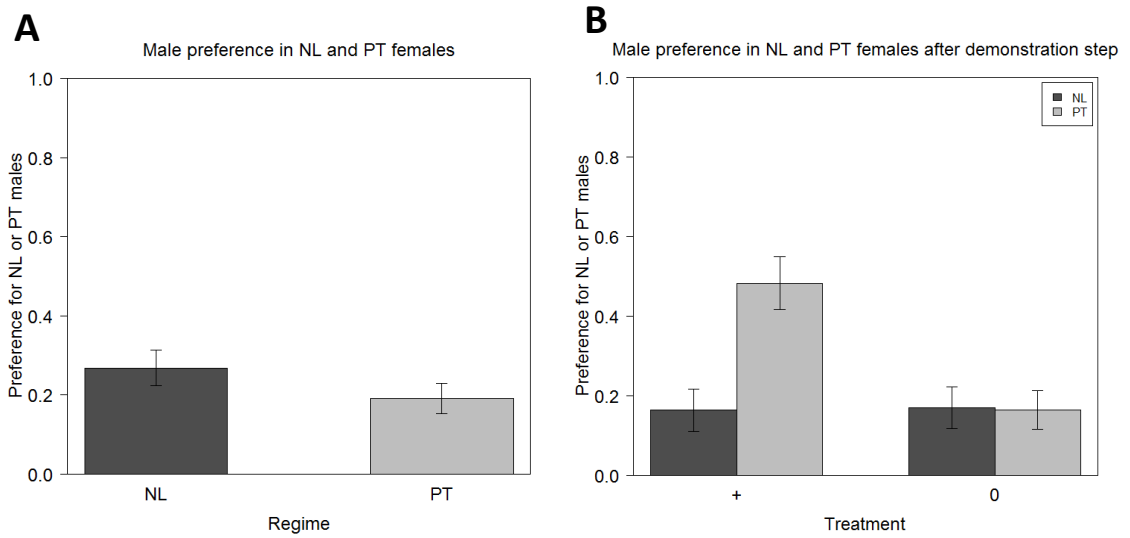

Figure 5. Female assortative preference for NL and PT males in the 10th generation. The preference of the NL and PT females were also analysed before (A) and after (B) the demonstration step at the 10th generation, in which females received positive social information about one type of male: + for the males from the females' own population and 0 for the males from the control population. Error bars represent the standard error of the mean defined with the difference between individuals.

After the demonstration step, NL females, similarly to generation 6, did not show any behavioural response to the social information received, meaning that there were no differences between treatments ($p=0.9294$; figure 5B; table S2), nor when comparisons are made between before and after treatment ($p=0.1123$ for + treatment and $p=0.3586$ for 0 treatment; figures 5A and 5B; table S3). Consequently, in both treatments females maintained their preference for males from the control regime ($p<0.001$ for both treatments).

In contrast, females from the PT regime responded to the social information but not in agreement with the results obtained in the 6th generation (figure 5B; table S2). In the + treatment, PT females showed MCC, increasing their preference for PT males when compared with the preference before treatment ($p=0.002$; table S3). Because of this, their preference for PT or TA males after the + treatment was not significantly different from 50% ($p=0.7929$). In the 0 treatment, PT females did not show differences before and after treatment ($p=0.7583$; table S3), maintaining the preference for males from the control regime ($p<0.001$). Therefore, there were significant differences between treatments ($p<0.001$). When comparisons are made

between the two generations, there is a significant interaction between generation and treatment ($p<0.001$) and differences between generations for all treatments ($p<0.001$) except for treatment + ($p=0.7401$).

Results from the mating step are also quite different from the 6th generation, since there was no significant assortative mating for any of the females for both regimes ($p=1$ for NL and $p=0.695$ for PT; figure 6A; table S4). Like in generation 6, there was no influence of social information (no effect of the treatment, $p=0.4069$) and no differences between NL and PT for this trait ($p=0.777$; figure 6A; table S4). When a comparison is made between the two generations, there are significant differences in females assortative mating both for NL ($p=0.01174$) and PT ($p<0.001$; figures 3A and 6A; table S4).

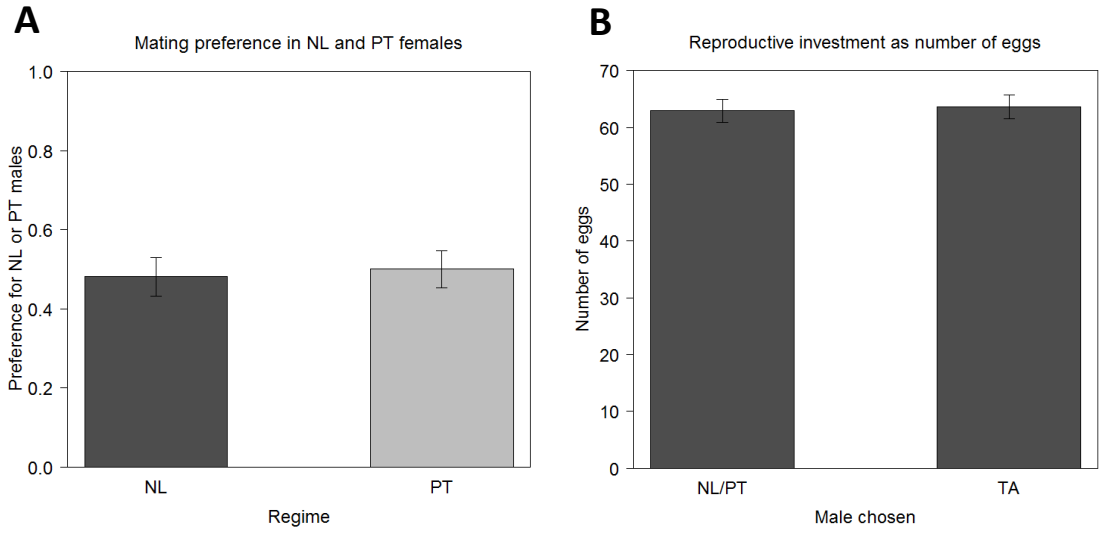

Figure 6. Female assortative mating and number of eggs laid in the 10th generation. The preference of the NL and PT females was also analysed in terms of mating preference in the 10th generation, after the post-demonstration step (A). The number of eggs laid by that same females was counted during three days and was used as one of the proxy of reproductive investment (B) and analysed in function of the male chosen in the mating step. Error bars represent the standard error of the mean defined with the difference between individuals (A) and between populations (B).

For the investment on the total number of eggs laid with each type of males, there is no difference between regimes ($p=0.764$; figure 6B; table S5), nor there is an effect of the social information received ($p=0.318$). A similar result was obtained when we compared the number of eggs laid when the matings occurred with different types of males (*Copulation* variable), with no differences between them ($p=0.764$). However, the total number of eggs did increase significantly between generation 6th to generation 10th, from an average of 51.6 and 48.1 to an average of 62.1 and 64 for NL and PT, respectively ($p=0.0251$ for NL and $p<0.001$ for PT; figure 3B and 6B; table S5).

The results obtained with total number of adults and juvenile viability also have different patterns from those found at generation 6 (figures 7A-D; table S6-9). Now there are no differences between the females that copulated with the males from the control regime and the

ones that copulated with the males from the NL or PT regimes, both for total number of adults (p=0.326; figure 7A; table S6) and juvenile viability (p=0.315; figure 7C; table S8). But now the model with the fixed factor *MCC* was not statistically different from the model with the fixed factor *Copulation* (p=1). We thus analysed the alternative reduced model with *MCC*.

For this variable, the significant values that were found are the differences with the control when females copulated with males for whom they received positive social information (the females that did MCC) (p=0.0208 for total number of adults and p=0.00818 for juvenile viability) and when they did not (p=0.00632 for total number of adults and p<0.001 for juvenile viability; figures 7B and 7D; table S7 and S9). When comparing the females that did MCC and those that did not, there were non-significant differences (p=0.7913 for total number of adults and p=0.3906 for juvenile viability). Differences between the two regimes were not significant either for this trait (p=0.3592 for total number of adults and p=0.213 for juvenile viability).

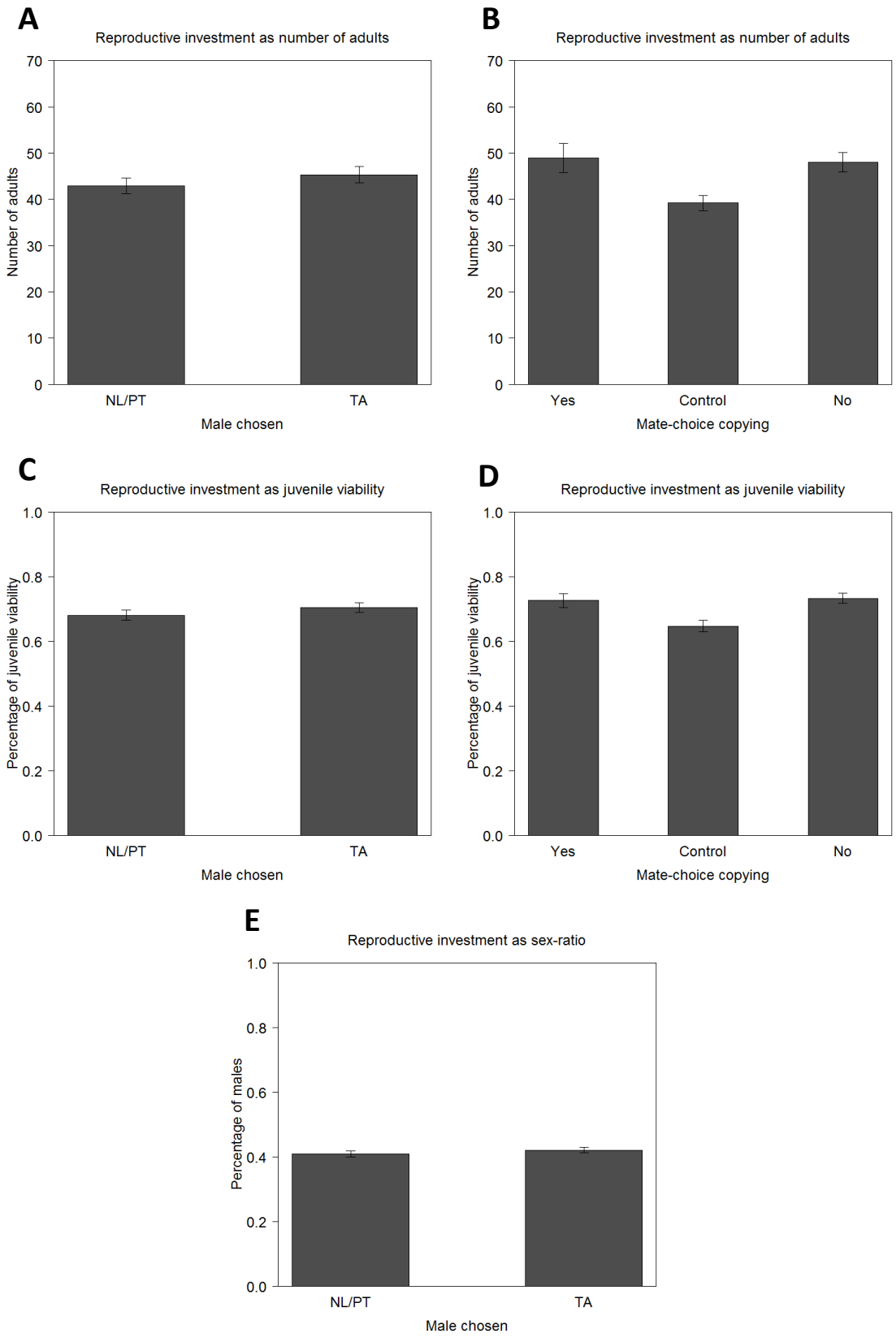

Figure 8. Traits related with female reproductive investment and mating choice in the 10th generation. The number of adults that emerged from the eggs that were laid during three days by the observer females (A and B), their juvenile viability (C and D) and their sex-ratio defined as percentage of males (E) in relation to the male chosen during mating step (A, C and E) and in relation to the occurrence/non-occurrence of MCC (B and D). Error bars represent the standard error of the mean defined with the difference between populations (A-D) and between individuals (E).

Similarly to what happened in the 6th generation, the sex-ratio at generation 10 (figure 7E; table S10) did not seem to be influenced by the regime from where females come from ($p=0.8018$), nor between females that chose NL/PT and females that chose TA ($p=0.267$). The social information had no effect either ($p=0.063$). The sex-ratio is biased towards females being approximately 59% for both regimes ($p<0.001$ for both of them).

These differences between the 6th and the 10th generation suggest an evolutionary response, which justifies a comparison between generations. For total number of adults, differences in the factor Copulation were statistically significant when females chose NL/PT ($p<0.001$), but not when they chose TA ($p=0.1261$). The interaction with generation was not significant ($p=0.1349$; figure 4A and 7A; table S6). For juvenile viability, the opposite occurs, being significant for TA ($p<0.001$) but not for NL/PT ($p=0.9308$), with a significant interaction between the variables ($p=0.0102$; figure 4C and 7C; table S8). Sex ratio showed an evolutionary response for both categories of females ($p<0.001$), although an interaction was not present ($p=0,124$; figure 4E and 7E; table S10).

For the variable MCC, the total number of adults was significant when females did not copy ($p<0.001$). Both the control females and the females that did mate-choice copy were not significantly different between generations ($p=0.7762$ and $p=0.0689$, respectively). However, the interaction between the two variables was significant ($p<0.001$; figure 4B and 7B; table S7). For juvenile viability, only the control has significant results ($p<0.001$) between generations. Both females that did MCC and the ones that did not have no significant differences ($p=0.0355$ but non-significant with FDR correction and $p=0.4507$, respectively). Again, the interaction between the variables was significant ($p=0.001$; figure 4D and 7D; table S9).

Discussion

Assortative preference and hierarchy of preferences

With this study, our main goal was to find out whether MCC in *Drosophila subobscura* can lead to the hybridisation of previously geographically isolated populations. We worked with *Drosophila subobscura* populations from the two extremes of the species European latitudinal cline – the NL (Netherlands) and PT (Portugal) regimes – and the simulated scenario was that of an immigration of PT and NL to a new (laboratorial) environment, with a resident TA population (adapted to the lab for several generations, since 2001). Was MCC going to facilitate the hybridisation of PT and NL with TA?

MCC in females is said to occur when they change their initial (probably innate) mating preferences about males of certain phenotypes for males with different phenotypes, as a response to the social information acquired by the mating choices of other females[20]. Measuring females' initial mating preferences is, therefore, an important step of the MCC experimental design. We found out, indeed, at generation 6, a strong initial assortative preference of females from both regimes, as well as, at generation 10, a strong initial preference for males of the locally adapted population (the control regime TA).

Besides indicating initial preferences, these results also show that these initial preferences do not rely exclusively on chemical cues provided by the males, but also on visual cues, given that the experimental design did not allow for chemical communication. To our knowledge, this is the first time that innate preferences in a species of *Drosophila* are shown to be based exclusively on visual cues. This is an interesting result, because it is generally assumed that chemical communication plays a more central role on species of *Drosophila*[55–58] than visual communication, that is thought to be important mostly to start courtship behaviours by males[59].

The fact that at generation 10 the females' initial preference changed for the males from the control regime, suggests that females are being strongly selected to choose males with a phenotype similar to the ones of the control regime – in other words, they are being selected to choose the more adapted males. This is very interesting because individuals from different regimes are being kept separated, which indicates that evolutionary convergence is happening to some degree.

Due to the fast change in females' assortative preference, we should assume that intersexual selection plays a significant role in *Drosophila subobscura* sexual interactions. Yet,

the mating step of these assays suggested the opposite. In both assays, females showed a clear preference for a certain male phenotype, both in the pre-demonstration and post-demonstration steps (except for the + treatment in PT regime, see further below), but these preferences did not seem to subsequently affect female mating choices during the mating step. Here, in the first assay, males from the control regime (TA males) were responsible for most of the matings when the preference of the females was in the opposite direction (except in the + treatment), and in the second assay the mating choice seemed to be random.

Independently of this, females' assortative preferences changed throughout the studied generations. One possible explanation is that females have different categories of preferences – innate preferences, social preferences, and preferences based on male performance –, giving priority to male performance over their innate and social preference when information is contradictory. Therefore, in the mating step of the first assay (generation 6), control (TA) males were responsible for most matings, because they were already adapted to the lab environment and, therefore, had better performance in intrasexual interactions. In the second assay (generation 10), both NL and PT males seemed already adapted, having the same performance than the control males and, because of that, female mating choices seemed to be random, when in fact the females were probably choosing the best of the two males that they were interacting with. However, in a scenario where a higher number of males are present (and this can happen both in nature and in the laboratory), it may be more difficult to access the relative performance of all males in intrasexual interactions. *Drosophila* species are likely to have a lek mating system [60] and, therefore, is easy to evaluate the relative performance of the males, but it is still harder than a scenario where only two males interact. So, it is likely that both female choice and male intrasexual competition play important roles in the mating outcome[61], but the protocol that we used in the mating step of our experiment did not allow to correctly isolate female preference from female evaluation of the males' performance. Besides male sexual performance, female mating decisions may also be disturbed by male-male competition. What we can take from here is that the post-demonstration step is crucial in a mate-choice design, particularly if male-male interactions cannot be avoided during the mating step and also if there is a lot of variance in the performance of the males that can be influencing the mating outcome.

Although male performance and female preferences are important, other factors could also be relevant. Females of *Drosophila subobscura* generally mate only once[62]. Thereby, they must ensure that the male with whom they choose to mate is the best one. But even if he is, if he had mated several times before, then he may not have an optimal quantity of sperm, thus not being a good choice[35]. In this scenario, females should avoid those males, even when they are preferred. This is a possible explanation for the behaviour of the PT females in the 6th

generation, because, after having received positive social information about the PT males, they subsequently avoided those males. This is compatible with the "sperm depletion hypothesis", as suggested by Loyau[35] in their work with *Drosophila melanogaster*, but was not expected in our case, because the males from the demonstration and post-demonstration steps were not the same individuals (see more details on this subject in the next section). Therefore, the performance of the males is probably the type of information that the females most likely use, since in the mating step the treatment does not seem to be relevant in defining their final sexual choice. An alternative explanation is that females are using chemical cues when in direct interaction with the males and so are able to understand that the males have not copulated yet, and thus choose based on male performance either way.

Finally, despite the lack of evidence for MCC in the 6th generation, the results clearly show that the social information that the PT females received had a significant effect in their behaviour during the post-demonstration step, providing evidence that the experimental protocol was adequate.

Individual recognition vs population recognition

Since the males were not the same between different steps, why should PT females avoid the males that already mated in the post-demonstration step? Plus this only happens in the first assay. Our hypothesis is that in the first assay the different males (PT and the control ones) had several phenotypical differences. Thus females might have focused on these discriminating differences instead of on individual ones, assuming thereby that the male was the same between experimental steps. However, by the 10th generation, females from both regimes started to prefer the control males, even though the control and the experimental regimes are kept separated in the lab and, hence, do not ever become in direct contact. As suggested above, this could be an evidence that PT and NL males were becoming similar to the TA males from the control regime. This is not due to changes in size, since a previous study concluded that males from the same geographical location of the PT regime do not change significantly during the same range of generations in the laboratory. Another observable phenotype may, thus, be changing in PT males during adaptation. If so, then, by the time of the second assay, PT and TA males were already more similar to each other due to convergence. On the other hand, size may still be important to NL individuals, since there are significant differences in this phenotype between populations from the extreme north of the cline and populations from the extreme south (with bigger size of the former), and these differences are maintained, at least for some generations, during adaptation to the lab.

According to this view, females, at the 10th generation, could now be focusing on individual differences, and no longer on population differences. Hence, they would now recognize that the males from the demonstration and post-demonstration steps were not the same and, because of that, they would not need to avoid sperm depleted males. Individual recognition has been found in *Drosophila melanogaster*[35,63], but the same was not yet studied in *Drosophila subobscura*.

Mate-choice copying and hybridisation

By the 10th generation, PT females did start to MCC. However, our results of the mating step in the first assay (generation 6) suggest that hybridisation is more likely to occur just due to male performance. MCC seems to become relevant only latter in the adaptation process to the laboratory conditions, after PT flies had first undergone, allopatrically, adaptive phenotypic changes to the modifications of their environment. Moreover, MCC occurred only within PT flies and not between PT females and TA males, thus not contributing to hybridisation, which goes against our hypothesis. This occurred probably because at generation 10, PT females' initial preference for TA males was already at 80%. It was difficult for MCC to increase this preference. This putts on evidence the fact that innate preferences evolved so fast during adaptation to the lab, that additional mate-choice processes for the local males became unnecessary. But, although MCC does not seem to increase hybridisation, it might be important within the population since it can contribute to define social preferences.

The fact that MCC only occurred at generation 10, when PT and TA males were more phenotypically similar, suggests, as written above, that at the beginning (generation 6) the males were too different for the females to rely on individual differences. They used population differences to reject the TA males and also did not detect that the PT males during the demo and post-demo steps were not the same individuals, thereby rejecting them in the post-demonstration step. At generation 10, TA and PT males were seemingly no longer identified with different populations and so individual differences became now the discriminating traits. This sequence of events suggests that MCC only occurs when females use individual discriminating traits, which is a totally new discovery, but also when the discriminating task is difficult, that is, when male mating quality is difficult to assess, which is already known from the literature[64]. Both conditions make the use of social information an important tool for female mate choice decisions. If it is true, then a preference generalization did occur since the individuals were not the same between steps. This is very exciting because MCC and its posterior generalization have been only observed in one species of invertebrate[32].

Preferences could have increased or decreased just because females had more visual interaction with one male type than with the other – the protocol included positive information only, but have should include a negative social information step as well. That was not included because the current protocol was already too time consuming and the whole set of steps had to be done in a single day, that is one day by each replicate population. Still, we believe that this does not apply to our data, since the response to the social information was different between regimes and, within the PT regime, between generations. It would be interesting, nevertheless, to see in what way negative social information would have changed the post-demonstration preference of the females.

Taken together, the results suggest that MCC is not needed for hybridisation to occur, because the better performance of the local males was enough. Besides, when MCC arises it might be contributing more to define social preferences within a population than between populations.

Differential allocation

After the mating step, we measured the reproductive investment of the females, looking for an effect of MCC in females' allocation of resources with the offspring of the copied males. We measured several fitness related traits, and found that differential allocation seems to be present, but only for juvenile viability and total number of adults. Neither number of eggs nor sex-ratio showed evidence of differential allocation and no evidence exists, as well, for a MCC effect in all four traits.

In the first assay, juvenile viability was only increased if females mated with control males for both NL and PT regimes. There are two alternative explanations for this: 1) females invested more with the male that had better performance; 2) control males are already adapted and, therefore, passed down genes that increased viability of the offspring. These two hypotheses are not mutually exclusive, but the data from the second assay supports the first one. Indeed, in the second assay, what seems to be relevant is if the females had the opportunity to choose between two males or not, since juvenile viability was higher in the experimental females (females that received the MCC treatments) relative to the control ones (females that were paired with one male only). Whether the females did MCC or not, or with which male they mated did not seem to matter.

Taken together with the data from the mating step, these results suggest that females are simply choosing the best males and increasing their own juvenile viability with them. This effect does not appear in the first assay because control males were better in general and,

therefore, juvenile viability only increased with them. By the time of the second assay, males seemed to have more similar performance and, consequently, the effect of the choice appeared.

There were also differences between generations for all these fitness-related traits. The number of eggs increased from the 6th to the 10th generation, but this was expected, because both regimes are adapting to the new environment[37]. However, the same did not happen to the number of adults and juvenile viability, in general. This is probably because development conditions between generations can be quite different – even in the lab –, giving rise to discrepancies that are only due to environmental differences and not genetic differences. Additionally, both development conditions were bad, so we are not confident that a comparison between generations is possible. Comparisons within the same generations are, on the contrary, robust because the conditions were the same for all individuals. Sex-ratio suffers from the same problem. This is likely the cause for the female sex-ratio bias in our data, since females are more resistant to environmental stress[65].

Differences between NL and PT

The only differences that are present between NL and PT are behavioural. This was not expected, because previous studies found differences in fecundity between populations from the same geographical locations of NL and PT, with those derived from the north having higher fecundity[37]. Assays of fecundity with our populations showed marginal significant differences between them, in the same direction (data not published). However, in those studies, fecundity was measured during the first 12 days after emergence, while in our study, we measured it during 3 days with a different protocol. Still, in this particular case, the aim was to see if there were differences in differential reproductive investment due to MCC. So a larger time frame would not have been particularly useful to this objective, since it is not expected that differential allocation would increase with more days of oviposition.

In what behavioural phenotypes are concerned, differences are present. Taking as a whole, females from the PT regime seem to have more flexibility in their preferences. If our hypothesis of individual recognition versus population recognition is correct, the fact that PT females, at generation 6, avoid males that they saw previously mating, suggest that PT males have, on average, less sperm than NL males. That would have created a selective pressure for PT females to avoid mated males. Differences in sperm length due to cline variation have already been found in *Drosophila melanogaster*[44]. A study in *Drosophila subobscura* found, however, no evidence of sperm length variation along latitudinal clines, nor significant differences between populations[66]. Nevertheless, this study was made with populations from North America (not

European ones) and does not mention differences in the quantity of sperm, but only in sperm length. NL and PT sperm differences can also be related to their sizes, since males of *Drosophila subobscura* from northern populations are usually bigger[67] and, therefore, might also store larger quantities of sperm.

Another important behavioural difference between NL and PT flies is that PT females seem to be able to do MCC, but NL females do not. We propose that this might be due to the fact that PT males have higher variability in fitness than NL, e.g. the natural environment of NL populations could be more prone to bottlenecks leading to less genetic variability between the males of this geographical location. This higher variability in fitness (including in the quantity of sperm) of PT males might then lead females to adopt a more plastic behaviour in mating preferences, which includes MCC generalization and avoidance of males that have mated in a recent past. Thereby, it would be useful to repeat this assay with other populations from different geographical locations to know if there really is a cline for this flexibility in *D. subobscura* sexual behaviour.

Either way, these results give strength to the importance of using individuals from different populations when studying MCC behaviour. Studies involving single populations might conclude that a species does not have MCC when, in fact, it might be that only a specific population does not have that behaviour, but other populations of the same species could have it.

Final remarks

Taking the results of these experiments all together, and taking into account the initial questions, two important discoveries have been made: (1) MCC is present in *Drosophila subobscura*, but only in one of two populations, putting on evidence the importance of the original ecological conditions for such a behaviour to be expressed; (2) MCC seems to be irrelevant to hybridisation, when fast adaptation to new environments involves changes on mating preferences that already facilitate hybridisation with the local population; and (3) that differential allocation is present in *Drosophila subobscura* at the juvenile viability level but MCC does not seem to have a role on this biological process.

Future studies should investigate these important discoveries more thoroughly.

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Appendix

Table S1 – Assortative preference in NL and PT females in the pre-demonstration step. For this analysis, the model fit was made with a binomial distribution. The significance levels of NL and PT assortative preference within generation 6 and generation 10 were obtained to see if they were significantly different from 50%. The other results are the possible comparisons that were made within the model, which includes the fixed factors *Regime* (NL vs PT) and *Generation* (6 vs 10).

Table S1		
Comparisons	**p-value**	**z-value**
NL - Generation 6	<0.001	4.669
PT - Generation 6	<0.001	5.737
Generation 6 - NL vs PT	0.46	0.738
NL - Generation 10	<0.001	4.382
PT - Generation 10	<0.001	5.953
Generation 10 - NL vs PT	0.188	1.317
NL - Generation 10 vs Generation 6	<0.001	6.403
PT - Generation 10 vs Generation 6	<0.001	8.266
Degrees of freedom: 400		

Table S2 - Assortative preference in NL and PT females in the post-demonstration step. For this analysis, the model fit was made with a binomial distribution. The significance levels of NL and PT assortative preference within generation 6 and generation 10 and within treatment + and treatment 0 were obtained to see if they were significantly different from 50%. The other results are the possible comparisons that were made within the model, which includes the fixed factors *Treatment* (+ vs 0), *Regime* (NL vs PT), and *Generation* (6 vs 10).

Table S2		
Comparisons	**p-value**	**z-value**
NL+ - Generation 6	<0.001	3.435
NL0 - Generation 6	<0.001	3.486
PT+ - Generation 6	0.4845	0.637
PT0 - Generation 6	<0.001	3.568
Generation 6 - NL+ vs NL0	0.4762	0.712
Generation 6 - PT+ vs PT0	<0.001	3.348
Generation 6 - NL+ vs PT+	<0.001	3.411
Generation 6 - NL0 vs PT0	0.8881	0.141
NL+ - Generation 10	<0.001	4.228
NL0 - Generation 10	<0.001	4.338
PT+ - Generation 10	0.7929	0.263
PT0 - Generation 10	<0.001	4.711
Generation 10 - NL+ vs NL0	0.9294	0.089
Generation 10 - PT+ vs PT0	<0.001	3.592
Generation 10 - NL+ vs PT+	<0.001	3.349
Generation 10 - NL0 vs PT0	0.9331	0.084
NL+ - Generation 6 vs 10	<0.001	5.545
NL0 - Generation 6 vs 10	<0.001	5.910
PT+ - Generation 6 vs 10	0.7401	0.332
PT0 - Generation 6 vs 10	<0.001	6.083
Generation*Treatment*Regime	<0.001	3.553
Degrees of freedom: 427		

Table S3 - Assortative preference comparison in NL and PT females between the pre-demonstration and post-demonstration step. For this analysis, the model fit was made with a binomial distribution. The results are the comparison between pre-demonstration and post-demonstration step in the degree of assortative preference of the NL and PT females. The model includes the fixed factors *Regime* (NL vs PT), *Differ* (Before vs After) and *Treatment* (+ vs 0). Analysis where only performed within each generation and not between generations.

Table S3		
Comparisons	**p-value**	**z-value**
NL+ - Generation 6 - Before vs After	0.4740	0.716
NL0 - Generation 6 - Before vs After	0.66907	0.427
PT+ - Generation 6 - Before vs After	<0.001	3.805
PT0 - Generation 6 - Before vs After	0.99798	0.003
NL+ - Generation 10 - Before vs After	0.11230	1.588
NL0 - Generation 10 - Before vs After	0.3586	0.918
PT+ - Generation 10 - Before vs After	0.00160	3.156
PT0 - Generation 10 - Before vs After	0.75827	0.308
Degrees of freedom: 468		

Table S4 - Assortative mating in NL and PT females in the mating step. For this analysis, the model fit was made with a binomial distribution. The significance levels of NL and PT assortative mating within generation 6 and generation 10 were obtained to see if they were significantly different from 50%. The other results are the possible comparisons that were made within the model, which includes the fixed factors *Generation* (6 vs 10) and *Regime* (NL vs PT).

Table S4		
Comparisons	**p-value**	**z-value**
NL - Generation 6	<0.001	3.734
PT - Generation 6	<0.001	4.382
Generation 6 - NL vs PT	0.5570	0.587
NL - Generation 10	0.695	0.392
PT - Generation 10	1	0
Generation 10 - NL vs PT	0.777	0.284
NL - Generation 6 vs 10	0.0117	3.394
PT - Generation 6 vs 10	<0.001	2.250
Generation*Regime	0.5282	0.631
Degrees of freedom: 409		

Table S5 – Reproductive investment as number of eggs. For this analysis, the model fit was made with a Poisson distribution. The results are the possible comparisons that were made within the model, which includes the fixed factors *Copulation* (NL/PT vs TA) and *Generation* (6 vs 10).

Table S5		
Comparisons	**p-value**	**z-value**
Generation 6 - NL/PT vs TA	0.712	0.37
Generation 10 - NL/PT vs TA	0.764	0.30
NL/PT - Generation 6 vs 10	0.0024	3.04
TA - Generation 6 vs 10	<0.001	3.32
Generation*Copulation	0.9569	0.05
Degrees of freedom:678		

Table S6 - Reproductive investment as number of adults. For this analysis, the model fit was made with a Poisson distribution. The results are the possible comparisons that were made within the model, which includes the fixed factors *Copulation* (NL/PT vs TA) and *Generation* (6 vs 10).

Table S6		
Comparisons	**p-value**	**z-value**
Generation 6 - NL/PT vs TA	0.00176	3.13
Generation 10 - NL/PT vs TA	0.326	0.98
NL/PT - Generation 6 vs 10	<0.001	3.34
TA - Generation 6 vs 10	0.1261	1.53
Generation*Copulation	0.1349	1.49
Degrees of freedom:657		

Table S7 - Reproductive investment as number of adults. For this analysis, the model fit was made with a Poisson distribution. The results are the possible comparisons that were made within the model, which includes the fixed factors *MCC* (Yes vs No vs Control) and *Generation* (6 vs 10).

Table S7		
Comparisons	**p-value**	**z-value**
Generation 6 - Yes vs No	0.0444	2.01
Generation 6 - Yes vs Control	0.284	1.07
Generation 6 - No vs Control	0.211	1.25
Generation 10 - Yes vs No	0.7913	0.26
Generation 10 - Yes vs Control	0.0208	2.31
Generation 10 - No vs Control	0.00632	2.73
Yes - Generation 6 vs 10	0.0736	1.79
Control - Generation 6 vs 10	0.7947	0.26
No - Generation 6 vs 10	<0.001	4.07
Generation*MCC	0.1349	1.50
Degrees of freedom:663		

Table S8 - Reproductive investment as juvenile viability. For this analysis, the model fit was made with a binomial distribution. The results are the possible comparisons that were made within the model, which includes the fixed factors *Copulation* (NL/PT vs TA) and *Generation* (6 vs 10).

Table S8		
Comparisons	**p-value**	**z-value**
Generation 6 - NL/PT vs TA	<0.001	4.342
Generation 10 - NL/PT vs TA	0.315	1.004
NL/PT - Generation 6 vs 10	0.9308	0.087
TA - Generation 6 vs 10	<0.001	3.762
Generation*Copulation	0.0102	2.569
Degrees of freedom: 658		

Table S9 - Reproductive investment as juvenile viability. For this analysis, the model fit was made with a binomial distribuition. The results are the possible comparisons that were made within the model, which includes the fixed factors *MCC* (Yes vs No vs Control) and *Generation* (6 vs 10).

Table S9		
Comparisons	**p-value**	**z-value**
Generation 6 - Yes vs No	0.131	1.510
Generation 6 - Yes vs Control	0.113	1.585
Generation 6 - No vs Control	0.889	0.140
Generation 10 - Yes vs No	0.3906	0.859
Generation 10 - Yes vs Control	0.0082	2.645
Generation 10 - No vs Control	<0.001	3.744
Yes - Generation 6 vs 10	0.0355	2.103
Control - Generation 6 vs 10	<0.001	3.477
No - Generation 6 vs 10	0.4507	0.745
Generation*MCC	0.0102	2.436
Degrees of freedom: 662		

Table S10 - Reproductive investment as sex-ratio. For this analysis, the model fit was made with a binomial distribution. The results are the possible comparisons that were made within the model, which includes the fixed factors *Copulation* (NL/PT vs TA) and *Generation* (6 vs 10).

Table S10		
Comparisons	**p-value**	**z-value**
NL/PT - Generation 6	<0.001	6.554
TA - Generation 6	<0.001	6.960
Generation 6 - NL/PT vs TA	0.279	1.083
NL/PT - Generation 10	<0.001	10.364
TA - Generation 10	<0.001	9.312
Generation 10 - NL/PT vs TA	0.267	1.109
NL/PT - Generation 6 vs 10	<0.001	11.688
TA - Generation 6 vs 10	<0.001	11.515
Generation*Copulation	0.125	1.536
Degrees of freedom: 652		